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SHORT REPORT

Combination of a monoclonal anti-phosphatidylserine antibody with gemcitabine strongly inhibits the growth and metastasis of orthotopic pancreatic tumors in mice

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Pancreatic cancer continues to have a dismal prognosis and novel therapy is needed. In this study, we evaluate a promising new target for therapy, phosphatidylserine (PS). PS is an anionic phospholipid located normally on the inner leaflet of the plasma membrane in mammalian cells. In the tumor microenvironment, PS becomes externalized on vascular endothelium. The monoclonal antibody 3G4 binds PS and promotes an inflammatory response against tumor blood vessels, resulting in reduction of tumor growth. Mice with orthotopic pancreatic tumors were treated with 3G4, gemcitabine or a combination of both drugs. Tumor burden including pancreas weight and metastatic lesions (liver, lymph node and peritoneal) were reduced 3- to 5-fold by the combination therapy as compared with 1.5- to 2-fold with 3G4 and gemcitabine alone, respectively. Treatment of tumor-bearing animals with the combination therapy increased macrophage infiltration into the tumor mass 10-fold and reduced microvessel density in the tumor by 2.5-fold compared with tumors from untreated animals. Gemcitabine alone and 3G4 alone were less effective than the combination of the 2 agents together. The additive therapeutic effect of both agents appears to be because chemotherapy increases PS exposure on tumor vascular endothelium and amplifies the target for attack by 3G4. In conclusion, 3G4 enhanced the anti-tumor and anti-metastatic activity of gemcitabine without contributing to toxicity.

Key words: vascular targeting; pancreatic cancer; metastasis; phosphatidylserine; monoclonal antibody; tumor microenvironment

Despite efforts to maximize the effect of surgery, radiation and chemotherapy, the 5-year survival for patients with pancreatic adenocarcinoma continues to be ~3%, the lowest of all malignancies (http://seer.cancer.gov). Even after surgical removal of a localized primary tumor, 70–80% of patients develop local, regional or distant recurrence.[1] Although the nucleoside analog gemcitabine (Gemzar, Eli Lilly and Co.) has the best activity against pancreatic adenocarcinoma, its use as a single agent has not improved the survival of patients with advanced pancreatic cancer.[2] In the present study, we explore the use of phosphatidylserine (PS) as a potential drug target for pancreatic cancer. PS is an abundant anionic phospholipid that is tightly segregated to the inner leaflet of the plasma membrane in most mammalian cell types.[3] The internal positioning of PS is maintained by an ATP-dependent transporter, aminophospholipid translocase.[4] Loss of PS asymmetry is caused either by inhibition of this translocase[5] or activation of PS-exporting enzymes[6] and is observed under a number of different pathologic and physiologic conditions (reviewed in ref 4, 5). Furthermore, malignant cells can expose PS in the absence of exogenous agents or cellular injury.[7] PS exposure is a general feature of 15–40% of tumor endothelium in all tumor models that have been examined.[8,9] Importantly, PS externalization on vascular endothelium is specific for tumor blood vessels and has not been found on blood vessels in normal tissues regardless of the model used.[9] Endothelial cells of PS-positive tumor blood vessels appear to be viable; they lack markers of apoptosis, are morphologically intact, and are functional at transporting solutes and blood.[10] Exposure of PS on endothelial cells in tumors is likely due to stress conditions in the microenvironment including cytokines, leukocytes, metabolites and hypoxia/reoxygenation conditions. These stresses elicit the production of reactive oxygen species, which may oxidize membrane phospholipids and generate calcium fluxes that inhibit aminophospholipid translocase and/or activate PS exporters. 3G4 is a murine IgG3 monoclonal antibody that binds anionic phospholipids complexed with β2-glycoprotein 1 and localizes to tumor blood vessels, as well as individual tumor cells and necrotic regions within solid tumors. In previous studies, 3G4 has shown significant anti-tumor effects as a stand-alone therapy and in combination with chemotherapy in multiple xenograft tumor models.[10,11] In the present study, we demonstrate that the combination of gemcitabine and 3G4 controls the growth and spread of metastatic pancreatic cancer in xenograft and syngeneic orthotopic mouse models.

Material and methods

Animals and tumor cells

Experiments were performed in athymic *nu/nu* mice (7–9-weeks-old, NCI, Frederick, MD) and C57BL/6 mice (7–9-weeks-old, Jackson Laboratories, Bar Harbor, ME). Animals were maintained according to the Institutional Animal Care and Use Committee of our institution and all procedures were performed following the Public Health Service Policy on Humane Care of Laboratory Animals. The human pancreatic adenocarcinoma cell line, MiaPaca-2 (obtained from the ATCC, Manassas, VA), was used in *nu/nu* mice, and the mouse pancreatic adenocarcinoma cell line, Pan02, was obtained from the Developmental Thera-

P. E. Thorpe and R. A. Brekken are consultants to and have equity interests in Peregrine Pharmaceuticals, Inc., which produces the anti-PS antibody, Tarvacin, and is sponsoring a phase 1 clinical trial of this antibody as an anti-cancer agent.

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peutics Program, NCI (Frederick, MD) and used in C57BL/6 mice. Both cell lines have been stably transfected with enhanced green fluorescent protein (eGFP).[12] All cells were maintained at 37°C in a mixture of 5% CO_2 and 95% air in Dulbecco's minimal essential medium (DMEM, Invitrogen; Carlsbad, CA) supplemented with 10% fetal calf serum (Gemini Bio-Products, Woodland, CA).

Antibodies

3G4 is a mouse IgG_3 antibody specific for PS-β2-glycoprotein 1 produced and purified in our laboratory.[10] Antibodies used for immunohistochemistry include monoclonal rat anti-mouse macrophage antibodies F4/80 (MCA497R, Serotec, Raleigh, NC) and rat anti-Mac-3 (clone M3/84, BD Biosciences, San Diego, CA), goat anti-PCNA antibody (sc-9857, Santa Cruz Biotechnology, Santa Cruz, CA) rabbit anti-active caspase 3 (AF835, R&D Systems, Minneapolis, MN), and monoclonal rat anti-mouse endothelial cell antibody MECA 32[13] (DSHB, University of Iowa, Iowa City, IA). Immunohistochemistry on acetone fixed frozen sections was performed and quantified as described.[14]

Orthotopic tumor model and treatment

Tumor cells were injected into the pancreas as described.[15,16] Briefly, animals were anesthetized using intraperitoneal anesthesia consisting of 1.2% ketamine HCl and 1% xylazine. The abdominal wall and peritoneum were open and the inferior pole of the spleen and tail of the pancreas was identified and externalized through the wound. Tumor cells (2×10^6 MiaPaca-2 or 5×10^5 Pan02 in 50 μl PBS) were injected underneath the capsule of the tail of the pancreas using a 30 gauge needle. Treatment was initiated 7 days after tumor cell injection with bi-weekly intraperitoneal (i.p.) injections of saline, 3.5 mg of gemcitabine (500 mg/M^2), 100 μg of 3G4 alone, or a combination of gemcitabine and 3G4. Treatment was continued in all mice until the experiments were terminated.

Animals were sacrificed when control mice showed adverse effects from tumor sequelae (weight loss, lethargy). At necropsy, liver metastases were quantified by identification of visible surface metastases. Lymph node and peritoneal metastases were quantified similarly by a thorough examination of the abdominal cavity. Final tumor weight was calculated in conjunction with the residual pancreas.

Statistical analyses

Statistical analyses were performed using SigmaPlot v9.01 (Systat Software Inc., Point Richmond, CA) with Student's *t* test where appropriate and with a Tukey-type multiple comparison test for proportions[17] as performed by the Center for Biostatistics and Clinical Science, UT-Southwestern Medical Center. Significance ($p < 0.05$) was determined with 95% confidence.

Results and discussion

The major findings to emerge from the present study are that a monoclonal anti-PS antibody, 3G4, enhanced the anti-tumor activity of gemcitabine against primary and metastatic pancreatic adenocarcinomas in mice.

3G4 alone or in combination with gemcitabine controls the growth of orthotopic pancreatic tumors

The final pancreas weights of mice bearing MiaPaca-2 pancreatic tumors (Fig. 1) were reduced by 69% ($p < 0.001$ *vs.* control) in mice receiving gemcitabine plus 3G4 as compared with a reduction in pancreas weight of 31% and 48% in groups receiving 3G4 or gemcitabine alone. Furthermore, the final pancreas weights of mice bearing Pan02 pancreatic tumors (Fig. 1) were reduced 61% ($p < 0.01$ *vs.* control) in mice receiving the combination therapy as compared with a reduction of 36% and 40% in groups receiving 3G4 or gemcitabine alone. In both mouse model systems the effect of the combination of gemcitabine with 3G4 was significantly better than either therapy alone, suggesting that gemcitabine enhances 3G4 function, or *vice versa*. Similarly, Huang *et al.*,[11] reported recently that 3G4 enhanced the inhibitory effect of docetaxel on the growth of breast tumors in an orthotopic mouse model. We also assessed cell proliferation and apoptotic cell death in representative tumors from each treatment group. Treatment with the combination therapy, gemcitabine, or 3G4 decreased the number of PCNA positive cells (Fig. 2) and increased the number of cells expressing active caspase 3 in the tumor mass (Fig. 2). The mean number of cells positive for active caspase 3 per high power field in tumors from animals treated with the combination therapy, gemcitabine, 3G4, or PBS was 23.1 ± 10.2, 20.9 ± 11.3, 23.9 ± 12.3 and 16.9 ± 8.7 respectively (mean ± SD, all treatments $p < 0.05$ *vs.* PBS). 3G4 as well as gemcitabine induce caspase activation; however, induction of tumor cell apoptosis is likely secondary to anti-vascular effects that result from 3G4 administration.

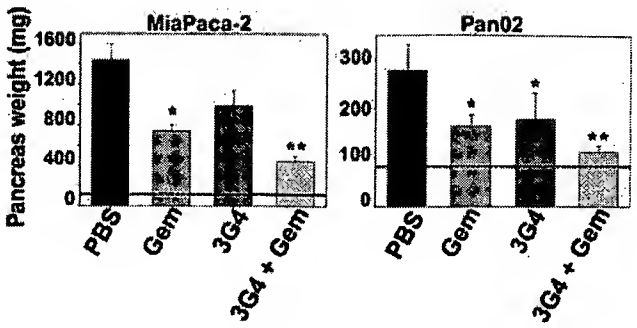

FIGURE 1 – Control of orthotopic pancreatic tumor growth by treatment with 3G4 combined with gemcitabine. *Nu/nu* mice bearing established orthotopic MiaPaca-2 pancreatic tumors or C57BL/6 mice bearing established orthotopic Pan02 pancreatic tumors were treated twice weekly with i.p. injections of PBS, 3.5 mg of gemcitabine (Gem), 100 μg of 3G4 (3G4) or 3G4 and gemcitabine (3G4 + Gem). The mean pancreas (normal pancreas + tumor) weight (mg) with standard deviation for each treatment group is displayed. The number of animals in each group of mice bearing MiaPaca-2 tumor was 12 except for the 3G4 + Gem group where $n = 11$ while the number of animals in each group of mice bearing Pan02 tumors was 6. The dashed line on each graph depicts the weight of a normal non-tumor bearing pancreas (≈90 mg) in a 20 g mouse. Significant differences of treatment groups *vs.* PBS group is indicated by *, $p < 0.05$; **, $p < 0.01$. Significant differences between treatment groups in mice bearing MiaPaca-2 tumors are as follows Gem *vs.* 3G4 + Gem, $p < 0.001$; 3G4 *vs.* 3G4 + Gem, $p < 0.005$. Significant differences between treatment groups in mice bearing Pan02 tumors were limited to the 3G4 *vs.* 3G4 + Gem, $p < 0.05$.

Combination therapy induces macrophage infiltration into tumors

Prior studies demonstrate increased macrophage infiltration into tumors treated with 3G4, and suggest this as central to the mechanism of action for anti-PS therapy.[10,11] Macrophage infiltration into MiaPaca-2 pancreatic tumors in animals receiving therapy was increased over control in all groups. Combination therapy increased macrophage infiltration 14-fold over control ($p < 0.01$), gemcitabine alone increased macrophage infiltration 4.2-fold over control ($p = 0.06$), and 3G4 increased macrophage infiltration 1.76-fold increase over control ($p = 0.22$) (Fig. 2).

PS is well described as an early marker of apoptosis and a facilitator of macrophage phagocytosis of apoptotic cells.[18] Apoptotic

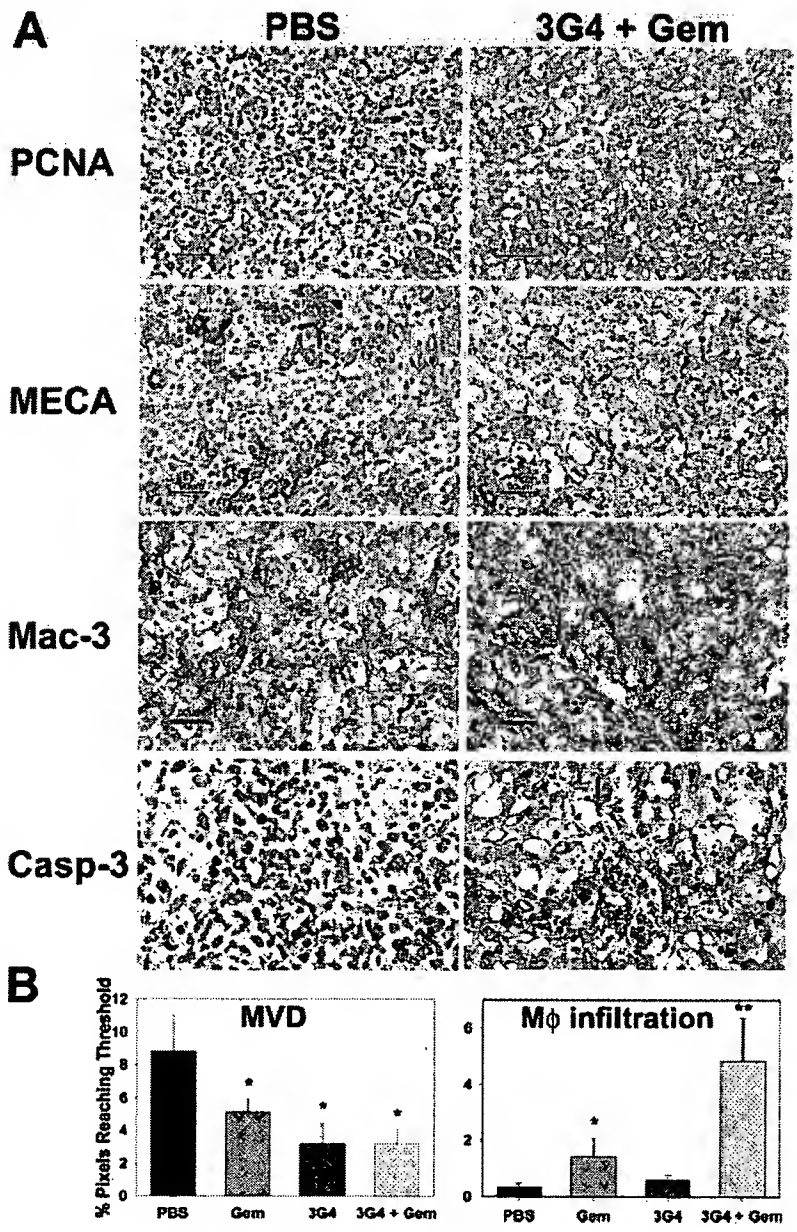

FIGURE 2 – 3G4 in combination with gemcitabine alters the tumor microenvironment. (*a*) Acetone fixed sections of MiaPaca-2 orthotopic tumors from each treatment group, PBS, gemcitabine (Gem, not shown), 3G4 (not shown), 3G4 + Gem, were analyzed for proliferating cells (PCNA), blood vessels (MECA), macrophages (Mac-3), and activated caspase-3 (Casp-3) by indirect immunohistochemistry. (*b*) Immunofluorescence for blood vessels (MVD) and macrophage (MΦ infiltration) in tumor sections from each treatment group was quantified by the use of Metamorph software. Five to ten fields for each treatment group were stained with either Meca 32 (MVD) or F4/80 (MΦ infiltration) and were captured using a Cool Snap CCD camera and the percentage of pixels exceeding a threshold determined. The total magnification for used was ×200. The threshold was set by determining the mean background levels using an isotype matched antibody of irrelevant specificity. The percentage of pixels reaching threshold is displayed (mean ± SD). Bars, 50 μm. *, $p < 0.05$; **, $p < 0.01$

cells are engulfed silently, without evoking an inflammatory response, whereas necrotic cells do evoke an inflammatory response. It is thought that PS on the surface of apoptotic cells signals a quiescence response by macrophage lineage cells in which TGF-β secretion is induced while secretion of TNF-α and IL-1 is suppressed. Necrotic cells on the other hand, do not signal quiescence, either because PS on necrotic cells is masked by normally intracellular PS-binding molecules (*e.g.*, annexin-5) or because proteases secreted by necrotic cells degrade the PS-receptor (PSR) on macrophages. Thus monocytes/macrophages that phagocytize necrotic cells default into the production of inflammatory cytokines, IL-1 and TNF-α. Because tumor endothelial cells are intact, viable, PS-expressing cells, it was proposed by Huang et al.,[11] that activated monocytes bind to PS-expressing tumor endothelial cells, but do not induce an inflammatory response against them. However, in mice treated with 3G4, PS on tumor endothelial cells is masked and is instead coated with antibody. Thus, macrophages attracted to the Fc portion of the 3G4 antibody promote a local inflammatory response, which leads to further immune cell recruitment and vascular damage, as well as destruction of tumor cells that expose PS. Gemcitabine is known to induce apoptosis in pancreatic cancer cells[19] and is expected to induce binding of 3G4 to both tumor cells and tumor endothelial cells.[11] Huang *et al.*,[11] demonstrated that docetaxel increased the exposure of PS on endothelial cells *in vitro* and resulted in increased localization of 3G4 to tumor endothelium *in vivo*. Thus a possible explanation for the enhanced therapeutic effect of treatment with 3G4 and gemcitabine is that gemcitabine potentiates 3G4 localization to tumor vasculature.

3G4 therapy alters tumor vasculature

The anti-tumor effects of 3G4 appear to be mediated at least in part by anti-vascular effects. Treatment with 3G4 or combination with gemcitabine decreased the density of MECA 32 positive blood vessels in pancreatic tumors by 63% and 64% (both $p < 0.05$), respectively (Fig. 2). These results are consistent with pre-

vious studies that show a reduction of blood vessel density and plasma volume within breast tumors treated with 3G4.[11] Another possible explanation for the enhanced anti-tumor activity of 3G4 combined with gemcitabine is that 3G4 targets tumor vasculature while gemcitabine targets the rapidly dividing tumor cells. Rapidly dividing tumor cells are typically found in the outer 1/3 of the tumor mass, the region of the tumor that is most difficult to destroy with a vascular targeting agent.

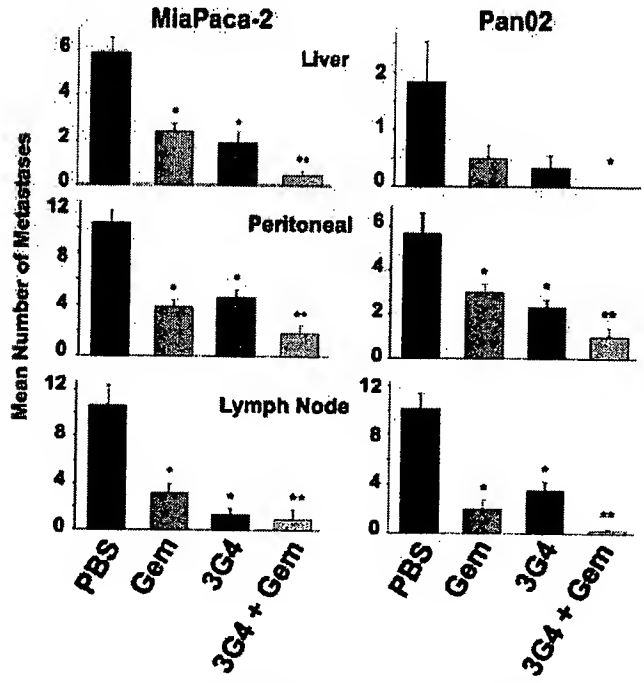

FIGURE 3 – 3G4 therapy alone and in combination with gemcitabine reduces metastatic burden in mice bearing orthotopic pancreatic cancer. Metastatic burden in *nu/nu* mice bearing MiaPaca-2 pancreatic tumors and C57BL/6 mice bearing Pan02 tumors was determined by counting visible tumor colonies on the organs at the time of sacrifice. The number of surface liver metastases (Liver) was determined by inspection of the tissue under blue light (485 nm) to visualize eGFP positive tumor foci. The number metastatic foci on the diaphragm, intestine and peritoneal wall (Peritoneal) and lymph nodes (Lymph Node) was determined in a similar fashion. The mean ± standard deviation of metastases per treatment group is displayed. Significant differences of treatment groups *vs.* PBS group is indicated by *, $p < 0.05$; **, $p < 0.001$. While significant differences between the treatment groups in mice bearing MiaPaca-2 tumors for the mean number of liver metastases were Gem *vs.* 3G4 + Gem, $p < 0.001$; 3G4 *vs.* 3G4 + Gem, $p < 0.01$; for the mean number of peritoneal metastases were Gem *vs.* 3G4 + Gem, $p < 0.02$; 3G4 *vs.* 3G4 + Gem, $p < 0.005$; for the mean number of lymph node metastases were Gem *vs.* 3G4 + Gem, $p < 0.005$. significant differences between the treatment groups in mice bearing Pan02 tumors for the mean number of liver metastases were Gem *vs.* 3G4 + Gem, $p < 0.05$; for the mean number of peritoneal metastases were Gem *vs.* 3G4 + Gem, $p < 0.005$; 3G4 *vs.* 3G4 + Gem, $p < 0.05$; for the mean number of lymph node metastases were Gem *vs.* 3G4 + Gem, $p < 0.05$; 3G4 *vs.* 3G4 + Gem, $p < 0.001$.

3G4 therapy reduces metastatic burden

The most striking finding of this study is that metastatic events were reduced significantly by 3G4 alone and in combination with gemcitabine in both the MiaPaca-2 and Pan02 tumor models (Fig. 3). In the MiaPaca-2 tumor model visible lymph node, peritoneal and liver metastases were reduced by treatment with gemcitabine alone, 3G4 alone, or combination therapy (all $p < 0.001$). Seventy-five percent of the control treated mice-bearing MiaPaca-2 tumors developed ascites in contrast to ascites formation in 8, 42 and 0% of gemcitabine, 3G4 and combination therapy treated animals, respectively (Table I). Of particular note is that the incidence of liver metastases dropped from 92% in the control-treated mice to 36% in the mice treated with both 3G4 and gemcitabine.

Immunocompentent C57BL/6 mice-bearing Pan02 tumors were also evaluated for metastatic burden (liver, lymph node and peritoneal) by examination of the abdominal cavity with the naked eye and using blue light (485 nm). There were striking reductions in metastasis formation at all of the principle sites of metastases in mice treated with gemcitabine plus 3G4. Visible lymph node, peritoneal and liver metastatic lesions were decreased significantly by treatment with gemcitabine, or 3G4, and to a greater extent by combination therapy (all $p < 0.001$). For example the percentage of mice having visible liver metastases was reduced from 83% in the PBS controls to 0% in the mice receiving both 3G4 and gemcitabine (Table I).

There were marked differences in the clinical course of the animals in this study. The control animals generally appeared ill at the end of the experiment, in contrast, the combination therapy group had normal weight gain and none of the combination treated animals developed ascites. Additionally, the animals that received combination therapy were well-groomed, active and showed no signs of distress. Furthermore, it is important to note that 3G4 did not increase docetaxel[11] or gemcitabine-related adverse effects. The lack of toxicity to normal vasculature of 3G4-based therapy is likely due to the specificity of PS exposure on tumor endothelial cells.[8]

Hematogenous metastatic dissemination of cancer is a highly selective, non-random process, consisting of a series of linked, sequential steps in which selected tumor cell(s) detach and are transported within the circulatory system.[20] During metastatic dissemination tumor cells are subjected to many stressors,[20] which we suggest induce PS exposure on disseminating cells. We hypothesize that PS positive tumor cells are targets for 3G4 within the vascular system and therefore are identified and cleared more efficiently by macrophages, Kupffer cells and reticuloendothelial cells within the liver and elsewhere.

Mice treated with 3G4 at the therapeutic dose (5 mg/kg, twice per week) did not display signs of toxicity. As previously reported,[10] the mice retained normal physical signs, coagulation

TABLE I – 3G4 IN COMBINATION WITH GEMCITABINE REDUCES METASTATIC INCIDENCE

Treatment	*n*		Incidence (%)[1]									
			Ascites		Lymph node		Peritoneal		Liver		Total[2]	
	Mia	Pan02	Mia	Pan02	Mia	Pan02	Mia	Pan02	Mia	Pan02	Mia	Pan02
PBS	12	6	9 (75)	2 (33)	11 (92)	6 (100)	12 (100)	6 (100)	11 (92)	5 (83)	12 (100)	6 (100)
Gem	12	6	1 (8)[a]	1 (17)	12 (100)	5 (83)	12 (100)	6 (100)	12 (100)	3 (50)	12 (100)	6 (100)
3G4	12	6	5 (42)	1 (17)	10 (83)	6 (100)	12 (100)	6 (100)	11 (92)	2 (33)	12 (100)	6 (100)
3G4 + Gem	11	6	0[a,c]	0	9 (82)	1 (17)[a,c]	7 (64)	4 (67)	4 (36)[a,b,c]	0[a]	9 (82)	5 (83)

[1]Metastatic burden determined by necropsy at the time of sacrifice, aided by visual inspection with blue light (485 nm) to visualize eGFP positive tumor cells.–[2]Total metastatic burden for the entire treatment group.The incidence of metastasis was compared between treatment groups were using a Tukey-type multiple comparison test for proportions.[17] Statistical significance was set at 5% such that a significant difference is indicated by a, *vs.* PBS; b, *vs.* Gem alone; c, *vs.* 3G4 alone.

variables, bone marrow cellularity, WBC counts and histology. Unengulfed apoptotic cells were not observed in normal tissues suggesting that 3G4 does not impair the clearance of apoptotic cells by the reticuloendothelial system. Indeed *in vitro* studies have shown that 3G4 promotes, rather than inhibits, phagocytosis of PS-expressing cells by macrophages and that macrophage recognition is mediated through the Fc region of 3G4 (Huang *et al.*, unpublished results). 3G4 thus appears to differ from other anti-cardiolipin antibodies that adversely affect coagulation, apoptotic cell clearance and self-tolerance.

Surgery provides the only improvement of survival in pancreatic cancer patients. However, few patients are eligible for surgical resection at the time of presentation and current chemotherapies do not enhance the ability to resect tumors. A chimeric version of 3G4, termed Tarvacin® has recently entered Phase I clinical trials for evaluation as an anti-cancer agent. It is hoped that translation of therapy consisting of Tarvacin with gemcitabine might provide an improved outcome for patients with metastatic pancreatic cancer.

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